An Investigation of the Mechanism Behind Adjuplex and its Effect in Adjuvant Combinations

A Thesis

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Gabriela Vogel

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Approved by:

Dr. Krishnendu Roy, Advisor
School of Biomedical Engineering
Georgia Institute of Technology

Dr. Balakrishna Pai
School of Biomedical Engineering
Georgia Institute of Technology

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Introduction

The Mechanism Behind Adjuvants

The immune system is characterized by two types of responses: the innate and the adaptive. The innate immune response is a quickly activated, general defense mechanism that deals with the identification and destruction of pathogens that enter the body and preventing them from spreading in the body. If the innate immune system is unsuccessful in destroying the pathogens, the adaptive immune system activates and follows with defenses that take a longer time to respond, but target pathogens more accurately.[3] The advantage of developing adaptive immunity is that the first time the body comes into contact with a pathogen it may take a few days but any following infection by the pathogen will be dealt with more quickly. Adaptive immune responses can react faster than those of the innate defense, if the antigen is already known, and an infection with this pathogen a second time around may have milder symptoms or may not even be noticed by the infected person. A technique biomedical engineers are researching into to increase the magnitude of adaptive immune responses from a vaccine and produce an effective form of immunity against specific pathogens, are the use of adjuvants.

Adjuvants are any substance that is formulated as part of a vaccine to enhance its ability to induce protection against infection.[3] Adjuvants have shown the ability to prolong antigen exposure to dendritic cells (DCs) and induce their maturation. DCs act as a messenger between the innate and adaptive immune systems and are essential in the propagation of the immune cascade. Mature DCs are cells designed to initiate contact with and activate surrounding immune cells such as T cells, natural killer cells, etc., which is required for the induction of potent and long-lasting immunity. In the event of a pathogen invasion, DCs detect invading microbes via toll-like receptors (TLRs). TLRs expressed on membranes of leukocytes (also referred to as

white blood cells), such as dendritic cells, detect by recognizing pathogen-associated molecular patterns (PAMPs) that are expressed on infectious agents. When TLRs are activated, DCs produce cytokines to induce the immune cascade for the development of effective immunity against the pathogen.[2]

Cytokines are chemical substances, such as chemokines, interferons, and interleukins, secreted by cells from the immune system that allow for cell-to-cell communication with one another to initiate and regulate the immune response.[9] For example, IFN-β is an interferon produced by dendritic cells that helps in detecting the effects adjuvants have by indicating when the TLRs have been stimulated and NF-κB is activated. To enhance the body's immune response to a pathogen, biomedical engineers are exploring adjuvants as an addition to vaccines to boost adaptive immune responses and elicit desired responses, including cytokine production. Utilizing adjuvants will aid in the development of improved, more effective vaccines that can aid in the production of a higher quantity of antibodies, the stimulation of more effective responses, and longer-lasting protection by activating TLRs on the dendritic cells.

Adjuplex Immune-Activation & Utilization

Adjuplex is a novel adjuvant comprised of lecithin and carbomer homopolymer.[11] Carbomers are a species of cross-linked polyacrylic acids that are already used in the biomedical industry as a stabilizer and thickening agent, and they demonstrate the ability to stimulate a strong T-cell response and a higher adaptive immune response than to an antigen alone. Lecithin is a biocompatible, naturally occurring surfactant found in biological membranes of things like soybeans or egg yolk. Lecithin also has beneficial properties as an emulsifier, stabilizer, and dispersing agent. Studies using Adjuplex show that when used as an adjuvant, it activates and alters antigen uptake, processing, and presentation. Additionally, Adjuplex has been shown to

trigger rapid leukocyte recruitment, pro-inflammatory cytokine secretion, and antigen capture by monocytes. Used alone Adjuplex has the potential to be an effective adjuvant.

It is important to observe the response the adjuvants produce when introduced to a system via vaccine because there can be issues with autoimmunity if T-cells are not regulated, or, as this research paper will later discuss, certain adjuvant combinations can have a subtractive response where one adjuvant may turn off the other adjuvant's response. Because adjuvants act on dendritic cells and induce effects that increase antigen immunogenicity, biomedical engineers are experimenting with different adjuvant doses and combinations to optimize the responses they produce and identify whether there is synergistic or suppressive immune-activation. Synergistic immune-activation is the phenomenon where the combination of two adjuvants that induce an immune response produce a higher response than they would individually. Suppressive immune-activation is the opposite phenomenon where the combination of two adjuvants that would individually produce an immune response would inhibit the response. Although it has shown to yield potentially synergistic adaptive immune response and elicit strong T- and B-cell response, there haven't been many studies looking at the interaction between Adjuplex and other adjuvants when combined.[5]

In addition, although Adjuplex displays several characteristics that classify it as an adjuvant, it has shown signs of toxicity at various doses at injection sites in mouse studies.[1,6] One way that we would like to deal with this issue and make Adjuplex more effective is to use particles as a delivery method. Nanoparticles and microparticles are a drug delivery method commonly used in pharmaceutical sciences and nanotechnology for encasing or carrying therapeutic agents to provide a targeted and controlled release therapy. Using nano/microparticles as a drug delivery system can have several benefits: they allow co-delivery

of antigens and adjuvants, enhance T-cell expansion and functionality, stimulate the formation of germinal centers, and assuage toxicity in vaccines.[9]

Collectively, we would like to look at different doses of Adjuplex to observe its toxicity, find an optimal dose of Adjuplex, observe cytokine production with the delivery of Adjuplex-adjuvant combinations, and experiment with the loading efficiency of these adjuvant combinations in nanoparticles for delivery and for vaccine optimization.

Literature Review

Adjuvants, substances added to boost the effects of vaccines, enhance adaptive immunity to experimental antigens by a variety of these mechanisms and pathways.[3] Adjuplex is a novel carbomer-lecithin-based adjuvant, and both the carbomer and lecithin aspects of Adjuplex aid in its classification as an adjuvant. Carbomers, a species of cross-linked polyacrylic acids with long and broad uses in biomedicine, have been evaluated as experimental adjuvants in veterinary vaccines. A report by Weggman demonstrates that biodegradable carbomers are not harmful to mammals and stimulate a more robust immune response than that with antigen alone.[11] Meanwhile, due to lecithin's properties as an emulsifier, stabilizer, antioxidant, and dispersing agent, it is utilized throughout the pharmaceutical industry for drug and vaccine delivery.[11] Both of these characteristics support the potential that Adjuplex has as a novel adjuvant for human vaccines, but there is some conflicting data on how effective Adjuplex can be in relation to or in combination with other adjuvants, implications of toxicity on the response, and the mechanisms behind Adjuplex's ability to produce an immune response.

Moreover, the discovery of toll-like receptors (TLRs) and the identification of TLRs as a signaling receptor for adjuvants has allowed for a better understanding of how adjuvants function

in regard to induction of innate and adaptive immune responses. With this, there are characteristics or responses that are used to characterize whether something is an adjuvant and to map out the mechanisms they use to achieve a response. For instance, retinoic acid-inducible gene 1 protein (RIG-I, also referred to as PUUC) participates in the recognition of single- and double-stranded RNA viruses and leads to the production of an appropriate cytokines and chemokines. Researchers can look at the release of cytokines and chemokines to identify whether RIG-I stimulates an antiviral state and drives the adaptive immune system toward an efficient, specialized response against an infection.

In addition, interferons, a subcategory of cytokines, and helper T-cells (Th1) can be used to look at long-term immune responses. Type I-IFN, activated by RIG-I, participates in NK cell activation, regulation of effector and memory T-cells, and B-cell activation for reoccurring or long-term exposure to an antigen.[8] Currently, some studies are being done to learn more about the immune response Adjuplex produces. In Weggman's research paper, they found that Adjuplex elicits a strong, balanced adaptive immune response and can drive potent antibody production that is protective against influenza virus challenge.[11] Also, when looking solely at the carbomer aspect of Adjuplex, carbomers induced its own form of systemic adjuvant activity through strong pro-inflammatory type-1 T-cell (Th1) polarization. They investigated potential pathways of immune activation by carbomers in comparison with another well-characterized adjuvant, and they concluded that carbomers may mediate its adjuvant activity via novel mechanisms. These mechanisms include antigen-presenting cell activation, Th1 induction, rapid leukocyte recruitment, pro-inflammatory cytokine secretion, and antigen capture by inflammatory monocytes.[4] Therefore, the constituents of Adjuplex alone prove to invoke an immune response which is what classified it as an adjuvant, however, Adjuplex has been

compared to only one or two commonly used adjuvants. Therefore, even though it produces an immune response, it is unknown to what degree it produces one along the spectrum of adjuvants.

On a larger scale, researchers can look at specific organs or the blood to make conclusions about the responses adjuvants have on the immune system as a whole. Results from a paper by Holbrook show that a vaccine with R848 adjuvant induces high-level of cell-mediated responses in primate neonates that result in increased virus clearance and reduced post-challenge compared with the nonadjuvanted virus vaccine. To make this conclusion, researchers obtained blood samples and analyzed antibody titers. This references the number of antibodies within the blood and it correlates with the strength of the immune response.[6] Additionally, cells can be isolated from organs, including lymph nodes, spleens, and bone marrow from bones. These cells can be used to look at T-cell activation and memory in response to a treatment and its effectiveness in creating an adaptive immune response. This technique was similarly used to characterize Adjuplex-induced immune response in a paper by Gasper. They reported that mice immunized with Adjuplex-supplemented intranasal vaccines generated cytotoxic T lymphocyte (CTL) responses and stimulated alterations in the frequency of dendritic cells in the lymph nodes. In addition, they reported that Adjuplex enhanced the ability of dendritic cells to promote an antigen-induced increase of naive CD8 T-cells by modulating antigen uptake, intracellular localization, and the rate of processing.[5] To classify how effective Adjuplex is as an adjuvant, a focus must be placed on recognizing the mechanisms that Adjuplex uses to produce an immune response throughout the entire body and to what degree. Researchers have looked at initial immune response but have failed to follow through and examine extensively the effects Adjuplex has on the immune cascade over an extended period across the entire body.

Furthermore, when looking at the risks versus benefits of adjuvants in vaccines, toxicity is a factor that is considered due to the damage it can cause to local tissue and the potential for inflammatory reactions at injection sites. For example, Monophosphoryl lipid A (MPLA), a low-toxicity derivative of the lipid A region of lipopolysaccharide (LPS), is commonly used as an adjuvant in mice and has shown to induce a strong Th1 response, suggesting that MPLA improves vaccine immunogenicity by enhancing antigen presenting T- and B-cells. While the toxicity associated with LPS prohibited its potential clinical use, MPLA is being developed as a vaccine adjuvant and is promising due to its low toxicity. Results from a paper from Beatty observed there was toxicity at the site of injection at higher doses of Adjuplex.[1] This data indicates that the dose of Adjuplex plays a role in toxicity levels and in the efficacy of producing an immune response, but there currently is a lack of research on appropriate Adjuplex doses to be used in vaccines. Similarly, in Weggman's research paper, they indicated that Adjuplex is well tolerated in mice.[11] However, other than quick eye observations of the injection sites for irritation, there was no additional data evaluating Adjuplex's toxicity.

A potential solution to the toxicity issue with the use Adjuplex, which we will discuss later on in this paper, is the use of nano/microparticles (NPs/MPs). NP/MP delivery systems for antigens have shown to be very useful in conjunction with adjuvants. NPs/MPs loaded with adjuvants have shown the capability to promote both cellular and humoral immune responses. For instance, mice immunized with NPs and MPLA showed broader humoral responses and the combination lasted more than one year in mice at lower doses compared to conventional adjuvants. Also, the researchers from this paper examined several components of the B-cell response and found that NPs/MPs promoted germinal center (GC) formation at low doses of antigen, that GCs gravitated towards clusters of NPs accumulating in the draining lymph nodes

over time, and enhanced the expansion of follicular helper T cells (Tfh).[9] Therefore, the use of NPs/MPs can be a promising solution to toxicity issues with Adjuplex while also promoting desired adaptive immune responses.

Moreover, this paper will address the Adjuplex combined with NPs/MPs, but it will also observe the immune response produced by Adjuplex combinations with other known adjuvants. In cases of synergistic immune responses, studies have shown that combination adjuvants can produce the desired, boosted immune response by activating different pathways. In the case of Adjuplex, flow cytometry studies revealed that Adjuplex recruited dendritic cells (DCs), monocytes, and neutrophils based on cytokine and chemokine secretion from these cells. However, Adjuplex neither triggered classical maturation of DCs nor activated NF-KB from pathogen recognition receptors (PRRs), suggesting the use of a different mechanism or pathway from previously reported PAMP-activated innate immunity cascade pathways followed by other adjuvants.[11] Therefore, since Adjuplex follows other immune pathways than traditional adjuvants, it will be difficult to predict what immune responses can occur in combining Adjuplex with other adjuvants. For instance, in Holbrook's paper about R848, the addition of a second TLR agonist (flagellin) to R848, a TLR agonist itself, did not enhance vaccine protection, suggesting that combinations of TLRs that provide increased efficacy have to be determined experimentally.[6] As a result, there is the possibility that adding another adjuvant or agonist may not enhance protection as we intuitively think it will while Adjuplex's mechanism remains unclear.

The current study addresses the lack of knowledge there is on the implications of combining Adjuplex with other common adjuvants, dose-dependent toxicity, and the mechanisms that Adjuplex uses to produce an immune response as a novel adjuvant. Using in

vitro methods, this study expands on Adjuplex toxicity from variable dose quantities and documents a possible dose that is reasonable for usage in vaccines. Additionally, we assessed cytokine production using Adjuplex in combination with other known adjuvants to conclude whether the addition of Adjuplex can produce a stronger immune response that can compete with other tested adjuvant combinations. More importantly, synergy between Adjuplex and other adjuvants is determined since current literature has shown that Adjuplex produces immune responses by activating different pathways than other adjuvants. After looking at how much activation Adjuplex causes when introduced to a system, methods such as using particles as a delivery system were implemented to observe whether the use of particles assists in the activation of a stronger immune response. Finally, the study will provide information towards determining the best way to use Adjuplex to customize vaccines to their pathogens so that the vaccine will produce the response needed based on the disease or condition the pathogen causes.

Methods and Materials

RAW-Blue Cell Treatment

RAW-Blue cells were kept in a medium consisting of DMEM medium (containing 4.5 g/L glucose and 2mM L-glutamine), 10% characterized fetal bovine serum (FBS), 1% penicillin-streptomycin (Pen-Strep) and 100 μg/mL of Normocin. Cells were fed using media supplemented with 100 μg/μL of Zeocin. When cells reached 90% confluence, media was aspirated out of flask and cells were dislodged from the bottom of the flask to prepare for splitting. RAW-Blue cells were centrifuged, resuspended in media, counted using a Countess Cell Counter, and split into flasks or plated at the appropriate concentrations for experiments.

Cell Culture

All animal experiments were approved by The Institutional Animal Care and Use Committees (IACUC) at Georgia Institute of Technology (Atlanta, GA). Bone marrow-derived cells (BMDCs) were generated from bone marrow of C57 BI/6 mice. Briefly, BMDCs were

isolated from tibias and femurs of mice and cultured in RPMI 1640 Glutamax medium (Invitrogen, Carlsbad, CA) supplemented with 10% characterized FBS, Pen-Strep, sodium pyruvate, beta-mercaptethanol, and 20 ng/mL mouse granulocyteemacrophage colony-stimulating factor (GM-CSF) for 6 days. On day 2 and 4, the medium was replaced with new medium containing GM-CSF. On day 7, loosely adherent DCs were harvested and used for further experiments.

Cell Treatment

Harvested DCs are plated in a 96-well plate with 300,000 cells per well (150 uL per well). The plate is put to incubate in 37°C and undisturbed for at least 2 hours to allow cells to adhere. Formulations of adjuvants are made. For MPLA, the stock solution (1 mg/mL in DMSO) is diluted in media to a concentration of 10,000 ng/mL, and for CpG, the stock solution (1.05 mg/mL in DI H_2O) is diluted in media to a concentration of 10,500 ng/mL. NP RIG-I involved the making and loading of the nanoparticle. NP RIG-I was diluted to a 25% concentration. Adjuplex is diluted to a 4% concentration in media. To make the combination adjuvant or nanoparticle formulations, Adjuplex was kept at a 4% concentration in media and the other part of the forumulation came from the diluted stock solutions mentioned earlier. From those formulations, 50 uL were added to the cells in the 96-well plate, and left to incubate at 37°C for 24 hours.

Variable Loading of Nanoparticles

To load the nanoparticles, R848-PLGA NPs were measured out and resuspended in Na-P loading buffer to obtain a concentration of 5 mg/mL. The resuspended nanoparticles were sonicated in a bath sonicator for five minutes before adding the different amounts of adjuvant based on concentration. Then, the vials were rotated end-to-end for at least twelve hours before being used. RNA loading efficiencies for all nanoparticles ranged between 80-90%.

Microplate Reader

Analyses were performed using BioTek Synergy HTX Multi-Mode Microplate Reader. In preparation for reading, RAW-Blue cells were rinsed in sterile PBS and diluted in test medium. Test medium contained RAW media with Normocin, Zeocin, heat-inactivated fetal bovine serum (FBS), and Penicillin Streptomycin (Pen-Strep) as instructed in RAW-Blue cell

datasheet for cell maintenance and activation. Diluted formulations of RAW-Blue cells and nanoparticle samples were added to a flat plate with QUANTI-Blue substrate provided with RAW-Blue cells and incubated for thirty minutes to stimulate activation. The plate was read and analyzed using Gen5 software at 24-, 48-, and 72-hour time points. This was also used to take the IL-18 and IL-1β ELISA data. ELISA procedures are similar to Luminex.

Luminex

A Luminex assay with diluted samples of RAW-Blue cells from InvivoGen in San Diego, CA and nanoparticles loaded with R848 and varying amounts of PUUC was performed. Another Luminex assay with treated samples of BMDCs with several soluble adjuvants and soluble adjuvant combinations was also performed later on. Samples were either used as is or diluted 5-fold in BMDC media and placed in flat bottom plate with magnetic beads. Samples were plated to fill four wells or eight wells down the column (depending on the experiment) to observe variability between samples. Diluted detection antibodies, tertiary antibodies, and streptavidin-PE (SA-PE) were added according to guidelines provided in Invitrogen ProcartaPlex Mouse Basic kit. Then, magnetic beads were resuspended using reading buffer before being put into the machine. The assay was performed with the purpose of measuring the amount of interferon beta (IFN-β), a cytokine, produced as a result of TLR activation by adjuvants.

Flow Cytometry

On day seven, BMDCs were plated at 300,000 cells/ mL per well in a 96 well plate and treated with various soluble adjuvants. After 24 hours of treatment, the BMDCs stuck on the plate after the supernatant was removed were scraped, resuspended in FACS buffer, and transferred into FACS tubes. Samples were incubated in diluted FC-Block for five minutes and then fluorescent antibody markers for 30 minutes in the dark at 4°C. Finally, the BMDCs were washed with FACS buffer, incubated in Biolegend fixation buffer, and analyzed using a BD Accuri flow cytometer. FloJo software was used for analysis. Samples were gated, and compensation was applied. Once the desired population was isolated, percentages and mean fluorescence intensities were graphed.

Results

I. Synergistic Immune-Activation

RAW-Blue cells are derived from RAW 264.7 macrophages and express almost all TLRs. When adjuvants are introduced to the RAW-Blue cells, the activated TLRs induce signaling pathways which lead to the activation of NF-κB, an important component for controlling cytokine production. Along with the activation of NF-κB, they secrete a secreted embryonic alkaline phosphatase (SEAP) gene which, combined with QUANTI-Blue substrate, makes the RAW-Blue cells turn blue and allows easy detection and measuring as shown in Figure 1 and 2. Using RAW-Blue cells, we looked at what synergistic activation TLRs influenced by adjuvant dose quantities and common adjuvant combinations. This concept is established now and used later on to defined whether Adjuplex produces a synergistic immune response.

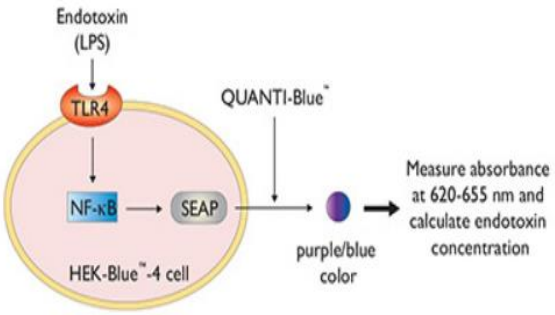

Figure 1: Diagram illustrating the cascade of activation within a RAW-Blue cell similarly to when an adjuvant is introduced to the system

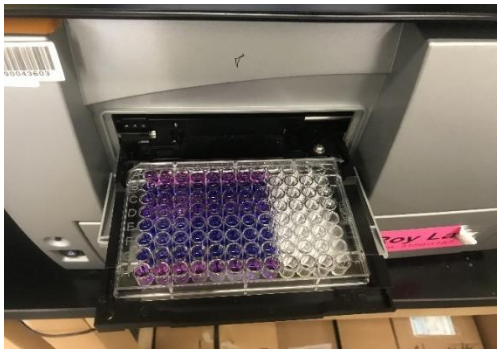

Figure 2: Picture showing example of plate layout and blue color after assay incubated with QUANTI-Blue

Influence of NP- R848-PUUC Loading Dose on NF-κB Production

Since NF-κB is an indicator that the adjuvant activated TLRs on the RAW-Blue cells, different doses of PUUC loaded in NP-R848 were tested to explore the effect increasing

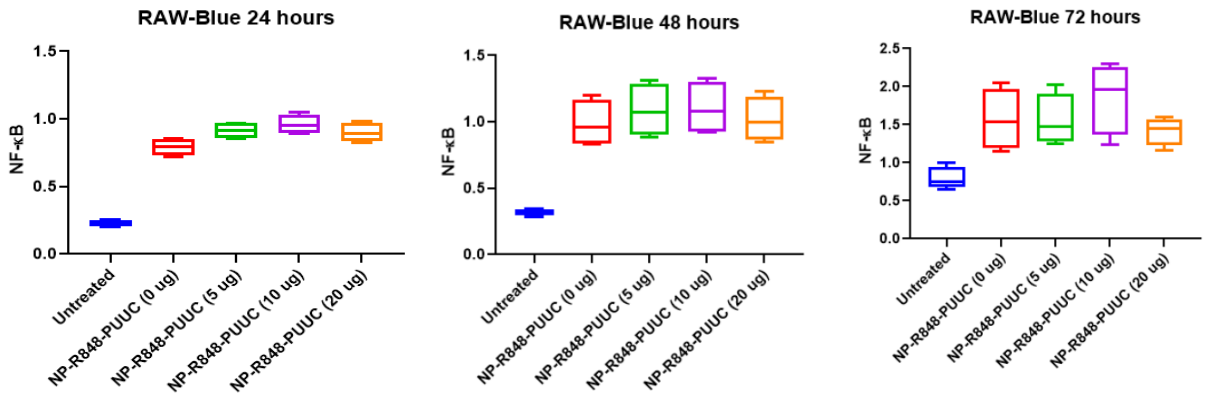

Figure 3: Results from 24-, 48-, and 72-hour time points of RAW-Blue cells NF-κB production. Each bar represents a well in the 96-well plate. Each plate had four samples of the same dose.

increments PUUC loaded on NP-R848 would have on TLR activation in the cells. The RAW-Blue cells with the nanoparticles were incubated in QUANTI-Blue substrate and run through the microplate reader to measure the SEAP levels, indicated by the absorbance or how blue each well turned. T-tests were performed to quantitatively analyze differences between the untreated control and the loaded NPs impact on NF-κB production. All samples displayed an increase in NF-κB production as shown in Figure 3. The mean value for the NP-R848-PUUC with 10 μg was the highest among all the doses. The two-tailed p-values ($p<.05$) indicate there was a statistically significant increase from the untreated after 72 hours. Therefore, we should expect to see an increase in TLR activation for adjuvant combinations that exhibit synergistic immune effects.

Different Formulations of Adjuvants in Nanoparticles and its Effect on NF-κB Production

Adjuvants are used to activate specific TLRs present in dendritic cells. Since no single adjuvant can induce all the protective immune responses, combinations of adjuvants have the potential to induce synergistic enhancement of immune responses that can be tailored to specific antigens found on pathogens. To explore this, RAW-Blue cells with NPs containing single, dual, and triple adjuvant combinations were incubated with QUANTI-Blue substrate and run through the microplate reader at 24- and 48-hour time intervals. T-tests showed that there were statistically significant increases ($p<.05$) between the untreated and NP-R848, NP-R848-PUUC, NP-R848-PolyIC, and NP-R848-PUUC-PolyIC (Figure 4). The p-values were similar between the NP-R848-PUUC, NP-R848-PolyIC, and NP-R848-PUUC-PolyIC combinations all being

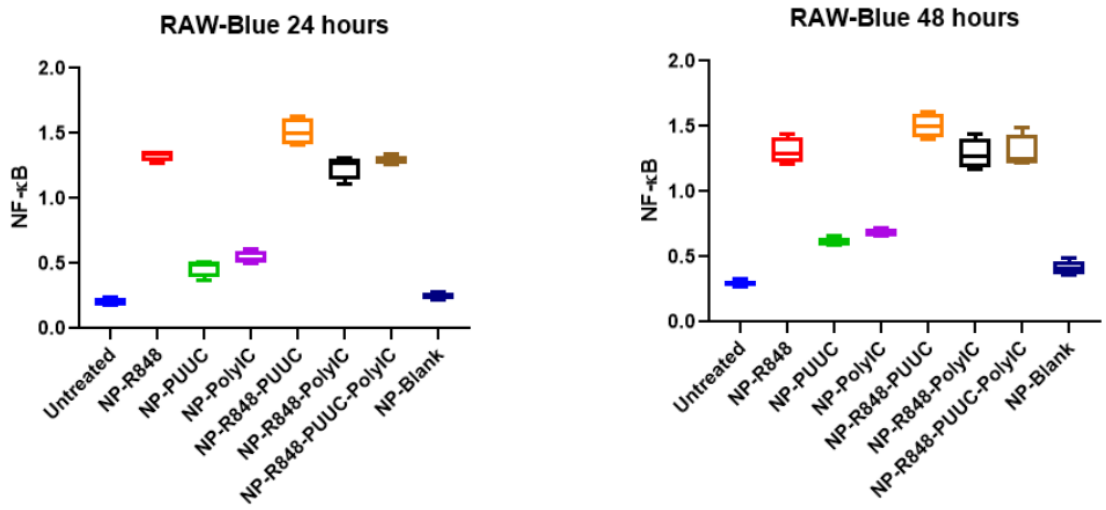

Figure 4: Results from 24- and 48-hour time intervals for RAW-Blue cells NF-κB production induced by different adjuvant combinations. Each bar represents a well in the 96-well plate. Each plate had four samples of the same dose.

around 0.0002. Overall, these results illustrate how combinations of adjuvants are capable of producing synergistic, boosted immune responses.

Measurement of IFN-β Production with Various NP-R848-PUUC Doses

When an adjuvant is introduced to a cell, the TLR is stimulated and NF-κB is activated. In addition, IFN-β is released out of the cell and can be measured by performing a Luminex assay. RAW-Blue cells were treated for 24 hours with NPs loaded with R848 and increasing increments of PUUC. Based on the data, there was an increase in IFN-β production in all the combination adjuvant samples. The NP-R848-PUUC with 20 μg had a larger spread than the other doses. The NP-R848-PUUC with 10 μg seems to produce the highest and most stable amount of IFN-β than the other doses (Figure 5).

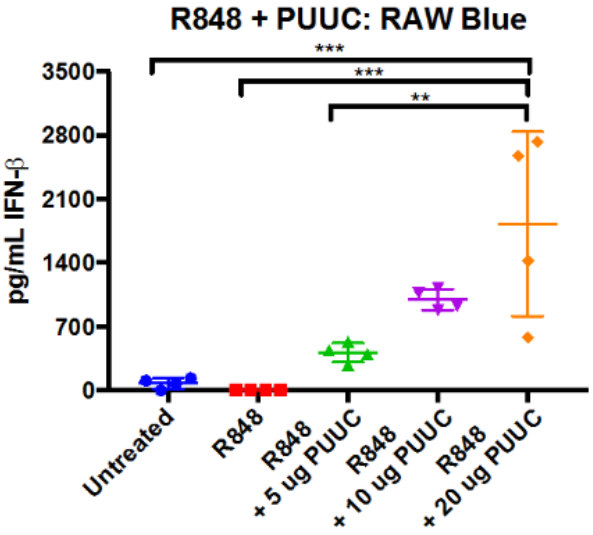

Figure 5: Graph shows the spread and individual points of IFN-β production of RAW-Blue cells

These results show that adjuvant combinations are dose-dependent, and this synergistic combination produced a boosted immune response.

IFN-β Production After Soluble Adjuplex, Soluble MPLA, Soluble CpG, and NP RIG-I Combinations Treatment

A Luminex assay was done with common adjuvants (MPLA and CpG), soluble RIG-I and nanoparticle RIG-I (NP RIG-I), and the newly obtained Adjuplex. Single adjuvants (soluble MPLA, CpG, and NP RIG-I) produced a response as expected, and the MPLA-CpG combination produced a higher amount of IFN-β than the single adjuvant treatments. This also correlates with previous data taken on these adjuvant combinations. However, Adjuplex alone and all combinations with Adjuplex produced almost no IFN-β (Figure 6). This was an unexpected result, and further testing was necessary to determine why these results were produced. Possibilities we theorized could be behind this was that the Adjuplex dose was too toxic and killed cells, which attributes to lower IFN- β production, or that the Adjuplex sample given to use may have expired.

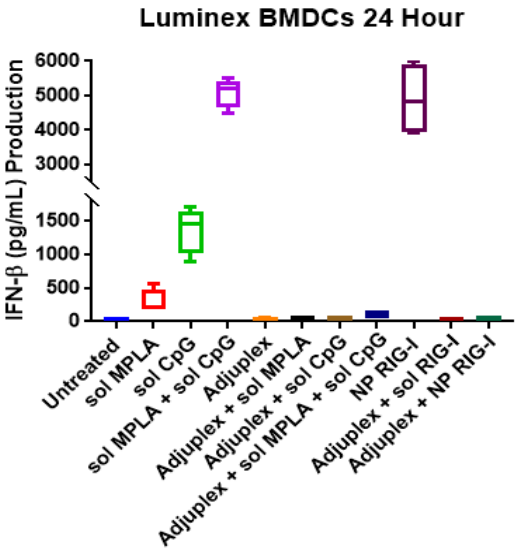

Figure 6: Graph of the spread and individual points of IFN-β production of the supernatant of each treated sample of BMDCs after 24 hours. Samples included: Adjuplex 9/18, Adjuplex 9/18 with combination adjuvants and nanoparticles, MPLA alone, CpG alone, MPLA and CpG, and NP RIG-I.

Soluble Adjuvant Treatment with Old and New Batch of Adjuplex

First, a Live/Dead Stain was performed to observe whether low IFN-β production was due to cell death from Adjuplex toxicity. Figure 7 shows that, even at the highest concentration of Adjuplex tested, only about 25% of cells were dead. Therefore, we could conclude that lower IFN- β production from above was not due to low cell count from toxicity. Additionally, since higher doses of Adjuplex yield better results according to literature, we decided to continue

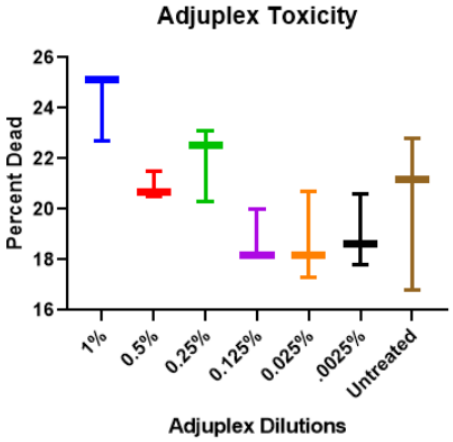

Figure 7: 7-AAD toxicity data

using the 1% concentration of Adjuplex in following experimentation. Next, collaborators who make the Adjuplex indicated that there could be a variation of toxicity between batches. Therefore, the Luminex assay for this round of experimentation included a batch of Adjuplex from October 2017 (10/17) and a newer batch from September 2018 (9/18). However, once again, single adjuvants (soluble MPLA and CpG) produced a response, the MPLA-CpG combination produced a higher amount of IFN-β than the single adjuvant treatments, and any combination with Adjuplex produced no amount of IFN-β (Figure 8). Therefore, we concluded there was no variation between the 10/17 batch and the 9/18 batch, and these theories could not provide a reason as to why Adjuplex was reacting this way.

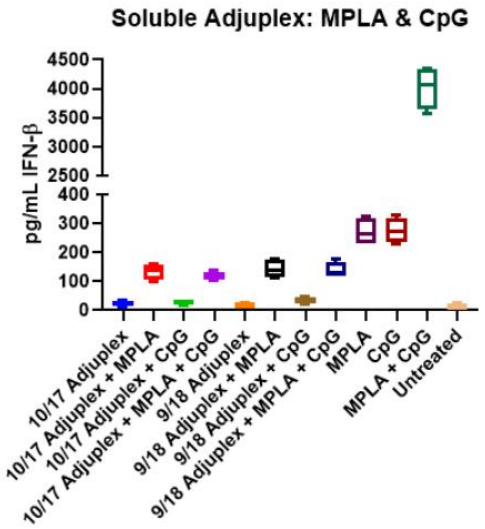

Figure 8: Graph of the spread and individual points of IFN-β production of the supernatant of each treated sample of BMDCs after 24 hours. Samples included: Adjuplex 10/17, Adjuplex 10/17 with combination adjuvants, Adjuplex 9/18, Adjuplex 9/18 with combination adjuvants, MPLA alone, CpG alone, and MPLA and CpG.

MTS Cell Proliferation Assay to Assess Cell Viability

An MTS assay is used to assess cell proliferation, cell viability, and cytotoxicity so conclusions can be made about how Adjuplex affects the cell metabolic activity. The supernatant of BMDCs treated with single, double, and triple adjuvant combinations after 48 hours. Overall, we concluded from the results, shown in Figure 9, that Adjuplex was not affecting the metabolic activity of cells nor killing cells.

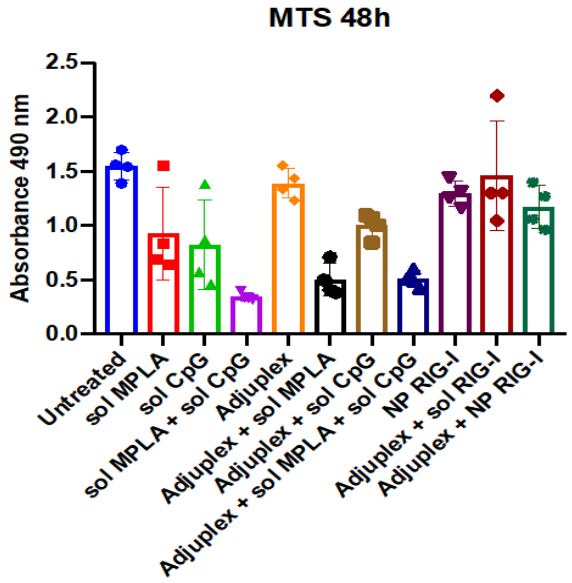

Figure 9: Graph of the spread and individual points of absorbance of each treated sample of BMDCs after 48 hours.

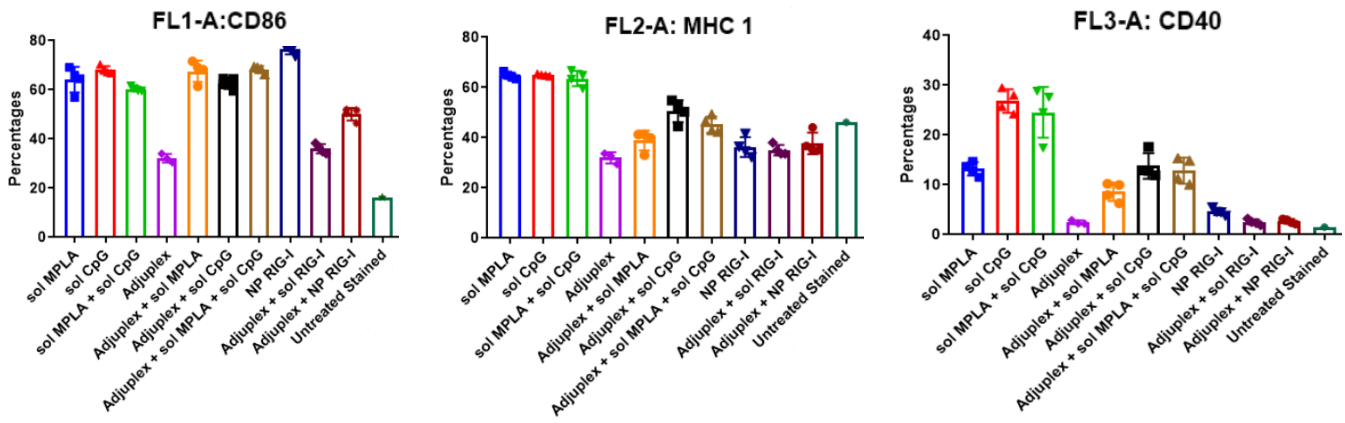

Figure 10: Graphs showing percentages of CD86, MHC I, CD40 protein expression taken from FloJo analysis software of samples.

Percentages of Costimulatory Proteins Detected After Soluble Adjuplex and Adjuvant Treatment

The treated BMDCs scraped from the wells and transferred to FACS tubes were analyzed by flow cytometry. The following markers were used to stain the cells: CD86, MHC I, and CD40 protein. CD86 protein expression indicates potential for T cell activation. MHC I molecule expression indicates an increased potential for MHC I presentation of antigen. CD40 induces downstream effects of the immune cascade. In comparison with the untreated stained sample, CD86, MHC I, CD40 expression in all samples treated with Adjuplex showed an increase (Figure 10). These results illustrate that Adjuplex is producing an immune response, but that the markers we have been looking at do not represent the pathway Adjuplex uses.

IL-18 and IL-1β Production with Adjuplex Combination Treatments

After doing some research into other pathways that Adjuplex may use to produce an immune response, we found the inflammasome pathway. An inflammasome is a sensor protein that can oligomerize into a pro-caspase-1 activating platform in response to PAMPs. The inflammasome pathway secretes proinflammatory cytokines, IL-18 and IL-1β. Therefore, to verify whether Adjuplex was activating the inflammasome pathway, we executed initial ELISA assays for these cytokines with Adjuplex and GLA, an adjuvant we had in hand. In both the IL-18 and IL-1β graphs in Figure 11, the results show that both adjuvants alone were not that strong, but, in combination, they produced a stronger immune response through the inflammasome pathway.

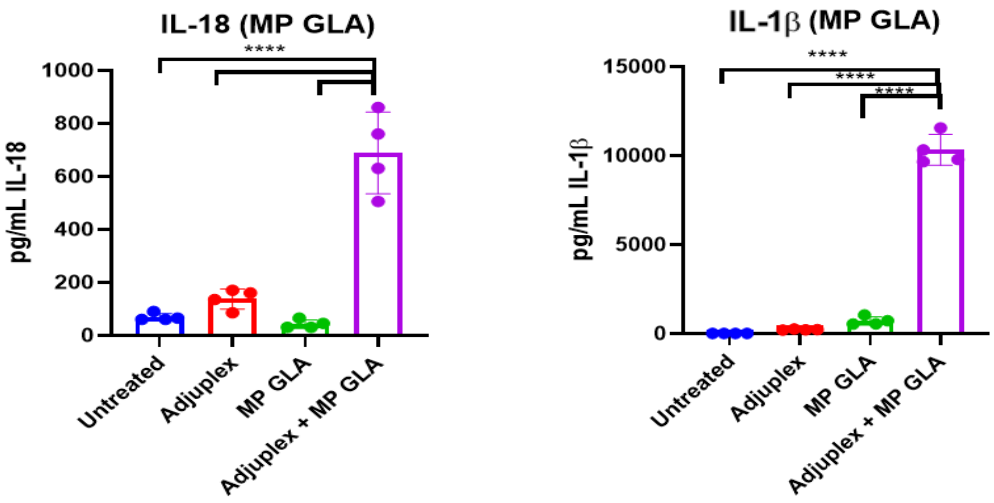

Figure 11: Graphs showing IL-18 and IL-1β production after treatment with adjuvants and adjuvant combination. This data was obtained from performing an ELISA.

Conclusions

Characterization of the type of adaptive immune response Adjuplex produces as an adjuvant is of critical relevance to the development of synergistic (and possibly suppressive) adjuvant combinations and optimization of vaccines. This thesis research contributed to the field of biomedical engineering by utilizing RAW-Blue cells to observe IFN-β and NF-κB production and make conclusions on the effects doses and combinations of adjuvants have on immune activation. In addition, Luminex assays, flow cytometry, and ELISA experiments were performed to examine Adjuplex, a novel adjuvant. From this data, we were able to learn more about the mechanism behind its ability to produce an immune response and how Adjuplex interacts with other adjuvants in combination.

The experiments presented provides insight in another procedural technique in assessing synergistic activation or suppression of TLRs via IFN-β and NF-κB production. RAW-Blue cells were used to look at double and triple adjuvant combinations and gave a basis on IFN-β and NF-κB production by adjuvants that were used to compare to the response Adjuplex produces. However, RAW-Blue cells still haven't been used to assess whether Adjuplex activates and, if so, to what degree it activates the NF-κB signaling pathway.

While investigations summarized in this thesis show that Adjuplex in combination with other common adjuvants such as MPLA and CpG decreases the production of IFN-β, this doesn't indicate that Adjuplex is blocking immune response that those adjuvants typically induce. We

were able to show that there was synergy between Adjuplex and GLA when looking at cytokines produced in the inflammasome pathway. Therefore, future work will expand on this finding and experiments will focus on further validating this finding by looking at other markers in the inflammasome pathway. Another experiment that can be done is to move to animal studies and treat mice with genes along the immune cascade knocked out to narrow down the pathway Adjuplex uses. Additionally, Adjuplex has only been compared to several common adjuvants and one particle formulation. In following experiments, we would like to expand on adjuvant combinations and particle formulations using Adjuplex.

References

1. Beatty, L., De, B., Havlicek, D., Kaminsky, S., Monette, S., Quach, L.,. . . Rosenberg, J. (2015). Safety of vaccination to treat cocaine addiction with capsid proteins from a disrupted adenovirus conjugated to a cocaine analog. *Immunological Aspects of Gene Therapy, 23,* S180-S181.
2. Coler, R. N., Baldwin, S. L., Shaverdian, N., Bertholet, S., Reed, S. J., Raman, V. S., … Reed, S. G. (2010). A Synthetic adjuvant to enhance and expand immune responses to influenza vaccines. *PLoS ONE, 5*(10), e13677. http://doi.org/10.1371/journal.pone.0013677
3. Coffman, R. L., Sher, A., & Seder, R. A. (2010). Vaccine adjuvants: Putting innate immunity to work. *Immunity, 33*(4), 492-503. doi:10.1016/j.immuni.2010.10.002
4. Gartlan, K. H., Krashias, G., Wegmann, F., Hillson, W. R., Scherer, E. M., Greenberg, P. D., . . . Sattentau, Q. J. (2016). Sterile inflammation induced by Carbopol elicits robust adaptive immune responses in the absence of pathogen-associated molecular patterns. *Vaccine, 34*(19), 2188-2196. doi:10.1016/j.vaccine.2016.03.025
5. Gasper, D. J., Neldner, B., Plisch, E. H., Rustom, H., Carrow, E., Imai, H., . . . Suresh, M. (2016). Effective respiratory CD8 T-cell immunity to influenza virus induced by intranasal carbomer-lecithin-adjuvanted non-replicating vaccines. *PLoS Pathog, 12*(12), e1006064. doi:10.1371/journal.ppat.1006064
6. Holbrook, B. C., Kim, J. R., Blevins, L. K., Jorgensen, M. J., Kock, N. D., D'Agostino, R. B., Jr., . . . Alexander-Miller, M. A. (2016). A novel R848-conjugated inactivated influenza virus vaccine is efficacious and safe in a neonate nonhuman primate model. *J Immunol, 197*(2), 555-564. doi:10.4049/jimmunol.1600497
7. Madan-Lala, R., Pradhan, P., & Roy, K. (2017). Combinatorial delivery of dual and triple TLR agonists via polymeric pathogen-like particles synergistically enhances innate and adaptive immune responses. *Scientific Reports*.
8. Martinez-Gil, L., Goff, P. H., Hai, R., Garcia-Sastre, A., Shaw, M. L., & Palese, P. (2013). A sendai virus-derived RNA agonist of RIG-I as a virus vaccine adjuvant. *J Virol, 87*(3), 1290-1300. doi:10.1128/jvi.02338-12
9. Moon, J. J., Suh, H., Li, A. V., Ockenhouse, C. F., Yadava, A., & Irvine, D. J. (2012). Enhancing humoral responses to a malaria antigen with nanoparticle vaccines that expand

Tfh cells and promote germinal center induction. *Proc Natl Acad Sci U S A, 109*(4), 1080-1085. doi:10.1073/pnas.1112648109

10. Wang, Y., Shi, J., Yan, J., Xiao, Z., Hou, X., Lu, P., . . . Qi, H. (2017). Germinal-center development of memory B cells driven by IL-9 from follicular helper T cells. *Nat Immunol, 18*(8), 921-930. doi:10.1038/ni.3788
11. Wegmann, F., Moghaddam, A. E., Schiffner, T., Gartlan, K. H., Powell, T. J., Russell, R. A., . . . Sattentau, Q. J. (2015). The Carbomer-Lecithin adjuvant Adjuplex has potent immunoactivating properties and elicits protective adaptive immunity against influenza virus challenge in mice. *Clin Vaccine Immunol, 22*(9), 1004-1012. doi:10.1128/cvi.00736-14